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# Simultaneous Determination of Phyllanthin and Hypophyllanthin in Herbal formulation by Derivative Spectrophotometry and Liquid Chromatography

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**ABSTRACT**

Two simple and accurate methods to determine Phyllanthin (PTN) and Hypophyllanthin (HTN) in herbal dosage forms containing *Phyllanthus niruri* extract, were developed using Zero order first derivative spectrophotometry and reversed-phase liquid chromatography (LC). PTN and HTN in herbal preparations (tablets) were quantitated using the Zero order first derivative responses at 259.2 nm for PTN and 252.4 nm for HTN in spectra of their solution in methanol. The calibration curves were linear with correlation coefficient, r = 0.9983 for PTN and 0.9977 for HTN in the concentration range of 10 to 50 µg/mL for PTN and 4 to 20 µg/mL for HTN. In the LC method, analysis was performed on a Phenomenex C-18 column (250 mm × 4.6 mm ID, 5 µm particle size), with isocratic elution using a mixture of tetrahydrofuran: water: acetonitrile in the ratio of 10:50:40 v/v/v at flow rate of 1 mL/min with UV detection at 230 nm. Both drugs were well resolved on the stationary phase, and the retention times were 16.05 minutes for PTN and 17.61 minutes for HTN. The calibration curves were linear (r = 0.9978 for PTN and 0.9996 for HTN) in the concentration range 10-100µg/mL for PTN and 5-50µg/mL for HTN. Both methods were validated, and the results were compared statistically. They were found to be accurate, precise, and specific. The methods were successfully applied to the estimation of PTN and HTN in herbal formulation containing *Phyllanthus niruri* extract.

**Key words:** Phyllanthin, Hypophyllanthin, Derivative spectrophotometry, Reversed phase liquid chromatography.

## INTRODUCTION

A number of species of *Phyllanthus* (Euphorbiaceae) are considered to be very effective hepatoprotective (1) agents in the Indian indigenous systems of medicine and are considered bitter, astringent, stomachic, diuretic, febrifuge, deobstruant and antiseptic. Still ayurvedic practitioners prescribe fresh juice of 'Bhuiamlaki' for jaundice. Various species of *Phyllanthus* are being sold in India under the trade name 'Bhuiamlaki' (2).

One of the most common species of Indian origin is that of *Phyllanthus niruri*, widely used as hepatoprotective and antigenotoxic (3). The plant contains different components such as flavonoids, terpenes, coumarins, tannins, saponins, alkaloids out of which lignan phytosterols, Phyllanthin (~ 0.5%) Hypophyllanthin (~ 0.2%) are highly active compounds which can serve as markers and used for the standardization of extracts and formulations (4).

Phyllanthin is (+) 3,4,31,41,9,91, Hexa Methoxy-8,8` bytyro lignan and hypophyllanthin is r-1-(3,4-dimethoxyphenyl) -6- methoxy-t-2, c-3-bimethoxymethyl-7-8-methylene dioxy-1,2,3,4-tetra hydro naphthalene (5). Literature reveals that UV-Spectrophotometric and liquid chromatography methods have been developed for the estimation of PTN individually in different parts of *P.niruri* (6,7). A number of High performance thin layer chromatography methods (HPTLC) are also available for the analysis of PTN and HTN simultaneously (8,9). High performance liquid chromatography methods using fluorescence detector (10) and UV detector for the estimation of Phyllanthus lignans have also been

reported but they lack sufficient resolution between PTN and HTN. Estimation of related compounds of Phyllanthin in plasma has also been reported using HPLC with fluorescence detection (11). Reports are available for estimation of the lignans by Micellar electrokinetic chromatography and HPLC-SPE-NMR (12,13) which are costly and not easily available.

This paper describes spectrophotometry and LC methods for the determination of PTN and HTN simultaneously. Also, the proposed methods are shown to be useful in determination of both markers in herbal preparations.

## MATERIALS AND METHODS

### *First Derivative Spectrophotometry*

*Instrumentation*

All absorption spectra and derivatives were recorded with a Shimadzu UV-1700 spectrophotometer with 10 mm matched quartz cells attached to a PC with UV Probe software Version 2.10 (Shimadzu, Kyoto, Japan).

### *Materials*

Authentic PTN and HTN were purchased from Natural Remedies, Bangalore, India. Nirocil tablets (Solumics Herbaceuticals, Bangalore, India) were procured from the local market. Each tablet contained extract equivalent to 1g of *Phyllanthus niruri*. Methanol of the analytical reagent grade, obtained by Qualigens (Mumbai, India) was used in the spectrophotometric studies.

### *Standard solutions*

*Stock solution:* PTN and HTN stock solutions (500 µg/mL) were prepared by weighing accurately 5mg of marker. The contents were transferred to a 10 mL volumetric flask and dissolved in methanol. The volume was made up using the same solvent. HTN stock solution was further diluted to the concentration of 100 µg/mL.

### *Selection of the wavelength for the estimation of PTN and HTN*

Standard solutions of PTN and HTN were diluted appropriately with methanol to obtain solutions containing 10 µg/mL of PTN and HTN. Spectra of these diluted solutions were scanned in the spectrum mode between 400 nm to 200 nm with a bandwidth of 2 nm and scan speed of 2400 nm/min vs methanol as a blank. These zero order spectra of PTN and HTN were treated to obtain corresponding first order derivative spectra with an inter-point distance of 10 nm in the range of 400-200 nm.

### *Derivative conditions*

The first-order derivative spectra of PTN and HTN were overlapped. The Zero-crossing point (ZCP) values of PTN at which the HTN showed some derivative response were recorded. The wavelength 259.2 nm was selected for the quantitation of PTN (where the derivative response of HTN was 0). Similarly 252.4 nm was selected for the quantification of HTN (where the derivative response of PTN was 0). Characteristic wavelengths (ZCPs) for PTN and HTN were confirmed by varying the concentration of both drugs.

### *Calibration Curves for PTN and HTN*

The standard solutions of PTN (500 µg/mL) and HTN (100 µg/mL) were used to prepare 2 different sets of diluted standard.

(a) Series A- This series consisted of PTN solutions of various concentrations (10-50 µg/mL) prepared by pipetting appropriate volumes (0.2, 0.4, 0.6, 0.8 and 1mL) of PTN standard solution into 10 mL volumetric flasks and diluting to volume with methanol.

(b) Series B- This series consisted of HTN solutions of various concentrations (4-20 µg/mL) prepared by pipetting appropriate volumes (0.4, 0.8, 1.2, 1.6 and 2 mL) of HTN standard solution into 10 mL volumetric flasks and diluting to volume with methanol.

### Method Validation

The method was validated for linearity, accuracy, precision, sensitivity, and stability by the following procedures:

### *Linearity*

Five different concentrations of PTN and HTN were scanned and their calibration curve was constructed in the specified concentration range (10-50 µg/mL for PTN and 4-20 µg/mL for HTN). The calibration plots were generated by replicate analysis ($n$ = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

### *Precision*

The interday and intraday precisions of the proposed first derivative spectrophotometry method were determined by estimating the corresponding response 3 times on the same day and on 6 different days over a period of 1 week for 3 different concentrations, 10, 30 and 50 µg/mL for PTN and 4, 12 and 20 µg/mL for HTN. The results are reported in terms of relative standard deviation (RSD) in Table 1.

### *Accuracy*

The accuracy of the method was determined by calculating recoveries of PTN and HTN by the method of standard additions. Known amounts of standards (80 %, 100% and 120%) were added to a pre-quantified sample solution. The amounts of PTN and HTN were estimated by measuring response at appropriate

wavelength 259.2 nm for PTN and 252.4nm for HTN. The recovery was verified by estimation of the markers in triplicate samples at each specified concentration level.

*Sensitivity*

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined by *kSD/s* where *k* is a constant (3 for LOD and 10 for LOQ), *SD* is the standard deviation of the analytical signal, and *s* is the slope of the concentration /response graph.

*Stability*

Solutions of both of the drugs in methanol were studied for their stability at ambient temperature for 24 h.

***Application to pharmaceutical preparation (Nirocil Tablets)***

The sugar coating of the tablets was removed and the tablets were ground to coarse powder. Approximately 100 mg of the powder was weighed and transferred to a 50 mL volumetric flask. 20 mL methanol was added to the flask and sonicated for about 20 minutes. The volume was made up when the extract dissolved completely in methanol to get a 1mg/mL solution. With the use of a marked capillary, 100 µL of the resultant solution was applied on chromatographic plate as a band along with a reference spot of standard PTN and HTN. Preparative TLC was run using silica gel G as a stationary phase and a mobile phase (8) consisting of n-hexane: ethyl acetate (2:1v/v). Visualization of PTN and HTN was performed under UV chamber, having Rf value 0.5 (Bright blue) and 0.53 (violet) for PTN and HTN, respectively. The bands of PTN and HTN were scrapped off using sharp blade, extracted with methanol (3 X 10mL) and filtered through Whatmann filter paper No.42 (Whatmann, London), the residue on the filter paper was washed with methanol and final volume of the solution was made up to 50mL. It was considered as a sample stock solution. 5mL of this solution was adjusted to 10 mL and used as samples.

***Liquid Chromatography***

*Instrumentation*

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20 µL fixed loop and a SPD-20A Prominence UV-visible detector. The separation was performed on a Phenomenex $C_{18}$ column (particle size 5 µm; 250mm X 4.6 mm ID; Phenomenex Torrance, USA) preceded by an ODS guard column (10 µm, 10mm×5mm ID) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd., Chennai, India).

*Materials*

Acetonitrile, tetrahydrofuran and water of HPLC grade were purchased from Qualigens (Mumbai, India). All the other solvents and reagents used were of analytical grade and were filtered through a 0.2 µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

*Preparation of Standard Solutions*

Combined standard solution of PTN and HTN- PTN and HTN standard (5mg each) were weighed and transferred to two 5mL volumetric flasks and dissolved in methanol. The volume was diluted to the mark with methanol. An aliquot of the solutions were pipetted out and transferred to a 10 mL volumetric flask, and volume was diluted to the mark with the mobile phase to obtain concentration of 400 µg/mL PTN and 200µg/mL of HTN (Solution PH).

*Chromatographic conditions*

Chromatographic estimation was performed using an equilibrated Phenomenex RP-C18 column (particle size 5 µm; 250mm X 4.6 mm ID), a mobile phase consisting of tetrahydrofuran: water: acetonitrile in the ratio of 10:50:40 v/v/v at flow rate of 1 mL/min with UV detection at 230 nm.

*Calibration curves for PTN and HTN*

Appropriate aliquot volumes (0.25, 0.5, 1.0, 1.5, 2 and 2.5 mL) from the standard solution PH were transferred to a series of 5mL volumetric flasks and volume in each flask was adjusted to the mark with methanol. The resulting concentrations of each drug in the flask were 10-100 µg/mL and 5-50µg/mL for PTN and HTN respectively.

***Application to pharmaceutical preparation (Nirocil Tablets)***

The sugar coating of the tablets was removed and the tablets were ground to coarse powder. Approximately 100 mg of the powder was weighed and transferred to a 50 mL volumetric flask. 20 ml methanol was added to the flask and sonicated for about 20 mins. The volume was made up when the extract dissolved completely in methanol to get a 1mg/mL solution. The solution was further diluted accordingly for assay.

**Method Validation**

The method was validated for linearity, accuracy, precision, robustness, sensitivity, system suitability and specificity by the following procedures:

***Linearity***

Six different concentrations of PTN and HTN were

analyzed and their calibration curve was constructed in the specified concentration range (10-100 µg/mL for PTN and 5-50 µg/mL for HTN). The calibration plots were generated by replicate analysis ($n$ = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

*Precision*

The precision was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of standard solutions at three concentration levels (10-5µg/mL, 40-20µg/mL and 100-50µg/mL). The intra-day assay precision was performed with the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

*Limit of detection and limit of quantification*

LOD and LOQ were determined by $kSD/s$ where $k$ is a constant (3 for LOD and 10 for LOQ), $SD$ is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

*Specificity*

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared binary mixtures. Moreover, the proposed method was applied to the proprietary formulation also.

*Robustness*

Robustness of the proposed method was evaluated by changing the column to a Hypersil ODS (particle size 5 µm; 250mm X 4.6 mm ID) column. The effect of change in solvent brand was also studied by using tetrahydrofuran and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India). Standard solution was injected 6 times for each change. System suitability parameters and RSD were calculated for each peak. Recoveries and % RSDs were calculated for each component during each change.

*Accuracy*

The accuracy of the method was determined by calculating the recoveries of PTN and HTN by the method of standard addition. Known amounts of the standards (80%, 100% and 120 %) were added to the pre-analyzed sample solution, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

## RESULTS AND DISCUSSION

***First derivative Spectrophotometry-***

**Figue 1** shows the UV spectra of PTN and HTN, which were found to be overlapping. Also PTN significantly contributes to the absorbance of HTN at the maximum absorbance wavelengths. Hence, the derivative graphical method was used to estimate PTN and HTN in presence of each other. The overlaid first-derivative spectra (D-1) of HTN and PTN were found to be appropriate for the determination of PTN and HTN by having separated ZCPs in methanol. The D-1 spectrum of HTN has zero absorbance at 259.2 nm, where PTN gives a significant derivative response, while the D-1 spectrum of PTN has zero absorbance at 252.4nm, where HTN gives significant derivative response. Therefore, 259.2 nm was selected for the estimation of PTN and 252.4 nm for estimation of HTN. It was also observed that with the increase in PTN concentration, the derivative responses at 259.2 nm increased.

The proposed First derivative zero crossing method showed linearity in the concentration range of 4 to 20 µg/mL for HTN and 10 to 50 µg/mL for PTN with correlation coefficient,0.9983 and 0.9977 for HTN and PTN respectively. The regression analysis of the calibration curves is shown in **Table 1**.

The precision result of the solutions at the three concentrations showed the %RSD values less than 2% both for intra-day assay and inter-day assay precision. The LOD values for HTN and PTN were 0.8014µg/mL and 0.1275 µg/mL respectively. The LOQ values for HTN and PTN were 2.4285µg/mL and 0.3864 µg/mL respectively (**Table 2**).

The average % recovery for PTN was 98.63 % and for HTN was 98.41 %, showing that the method does not suffer from any interference due to other constituents. Rigorous analysis of the results shows that the presence of other constituents in the formulation did not interfere with the final determination of active components (**Table 3**).

***LC method***

A simple HPLC method was adopted for the simultaneous determination of PTN and HTN either in binary mixture or in pharmaceutical formulation. To optimize the proposed HPLC method, all of the experimental conditions were investigated. For the choice of stationary phase, reversed-phase separation was preferred due to the drawbacks of the normal phase, eg., hydration of silica with water that can cause peak tailing.

To optimize the mobile phase, different systems were tried for chromatographic separation of the components by combining homogenous design and solvent polarity optimization. The best resolution was

*Table 1 : Regression analysis of the calibration curves for PTN and HTN for the proposed first-derivative spectrophotometry and LC methods*

| Parameter | First Derivative Spectrophotometry | | LC | |
|---|---|---|---|---|
| | PTN | HTN | PTN | HTN |
| Linearity range, µg/ml | 10-50 | 4-20 | 10-100 | 5-50 |
| Slope | 0.8154 | 0.7447 | 0.014 | 0.0064 |
| Standard deviation of slope | 0.0123 | 0.0119 | 0.00243 | 0.00965 |
| Intercept | 2.1567 | 5.4514 | 0.0065 | 0.007 |
| Standard deviation of intercept | 0.098 | 0.121 | 0.000396 | 0.000904 |
| Correlation coefficient (r) | 0.9983 | 0.9977 | 0.9978 | 0.9996 |

*Table 2: Summary of validation parameters for the proposed First derivative spectrophotometry and LC methods.*

| Parameter | First Derivative spectrophotometry | | LC | |
|---|---|---|---|---|
| | PTN | HTN | PTN | HTN |
| Limit of detection , µg/ml | 0.1275 | 0.8014 | 0.6295 | 0.342 |
| Limit of quantification, µg/ml | 0.3864 | 2.4285 | 1.907 | 1.038 |
| Precision (RSD%) | | | | |
| Intraday | 2.28 | 2.61 | 2.56 | 1.98 |
| Interday | 2.1107 | 1.41 | 0.95 | 1.44 |
| Repeatability (n=6) | 1.12 | 1.08 | 0.98 | 0.76 |

*Table 3 : Recovery study of PTN and HTN added to the preanalyzed samples using the proposed methods. (n=3)*

| Amount added µg/ml | | Recovery ,% ± SD | | Recovery ,%±SD | |
|---|---|---|---|---|---|
| | | First Derivative Spectrophotometry | | LC | |
| PTN | HTN | PTN | HTN | PTN | HTN |
| 11.68 | 11.53 | 98.14± 0.12 | 98.15 ± 0.28 | 99.09 ± 0.24 | 98.95 ± 0.46 |
| 12.98 | 12.82 | 98.02± 0.63 | 99.98 ± 0.72 | 100.23 ± 0.43 | 99.96 ± 0.96 |
| 14.27 | 14.10 | 99.24 ± 0.19 | 99.54 ± 0.44 | 98.02 ± 0.71 | 98.14 ± 0.85 |

*Table 4 : Results of system suitability parameters obtained from LC method*

| Parameters | PTN | HTN |
|---|---|---|
| Retention time (min) | 16.05 ± 0.1 | 17.31± 0.1 |
| Capacity factor (k') | 0.8947 | 0.9023 |
| Separation factor (α) | 1.009 | ----- |
| Efficiency/length(t.p/m) | 13888 | 13456 |
| HETP (mm) | 0.049 | 0.048 |
| Resolution (Rs) | 1.929 | 3.026 |
| Assymmetry (As) | 0.994 | 0.959 |

*Table 5 : Assay results of herbal dosage forms using the proposed first derivative spectrophotometry and LC. (n=3)*

| Formulation | PTN ± SD(µg/tablet) | | HTN± SD (µg/tablet) | |
|---|---|---|---|---|
| | First Derivative Spectrophotometry | LC | First Derivative Spectrophotometry | LC |
| Tablet (Nirocil) | 158.67±1.34 | 169.45±0.95 | 125.15±1.52 | 139.65±1.12 |

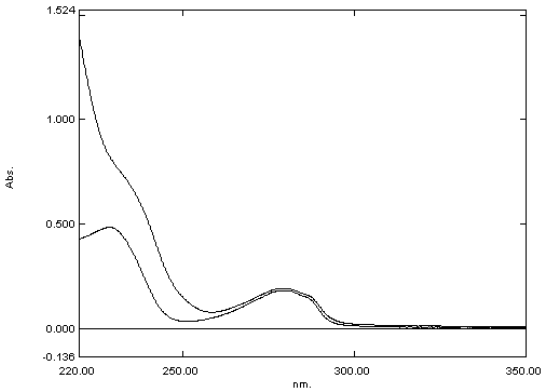


*Figure 1 Overlaid zero order absorption spectra of PTN (10µg/ml) and HTN (10µg/ml) in methanol.*

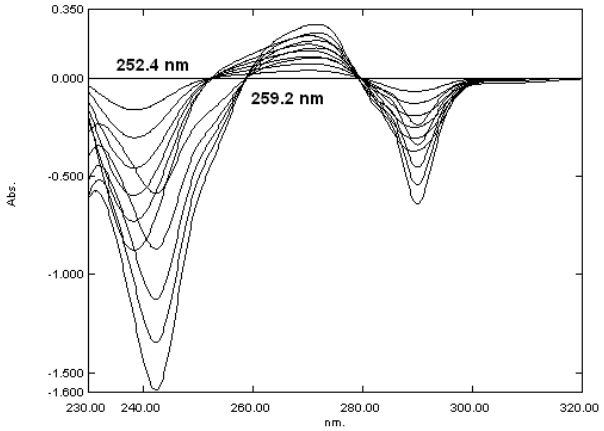


*Figure 2 Overlaid First Derivative absorption spectra of PTN and HTN.*

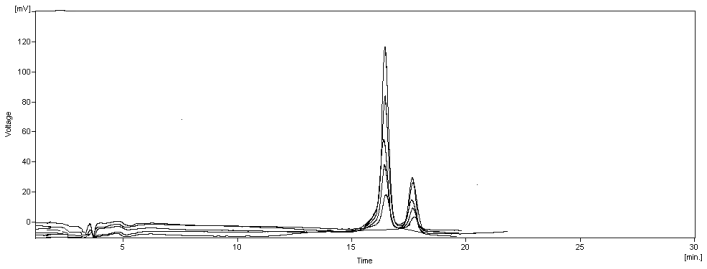


*Figure 3 Overlaid Chromatograms of PTN and HTN.*

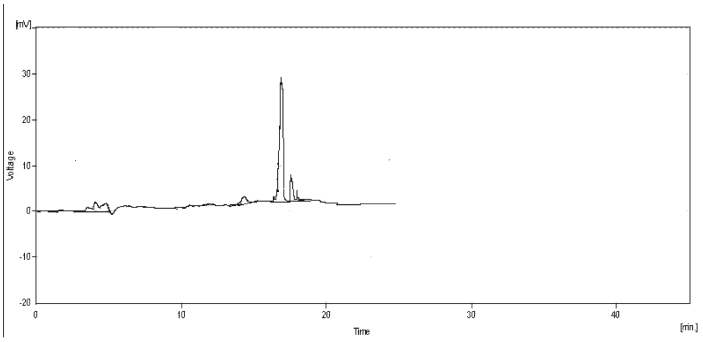


*Figure 4 HPLC Chromatogram of Nirocil tablet.*

achieved using a mobile phase consisting of tetrahydrofuran: water: acetonitrile in the ratio of 10:50:40 v/v/v, which gave good resolution and sensitivity of both drugs (**Figure 3**).

The calibration curves (n=3) constructed for the markers were linear over the concentration range of 10-100 µg/ml for PTN and 5-50 µg/ml for HTN. Peak areas of the markers were plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.9978, 0.9996 for PTN and HTN respectively with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (**Table 1**).

The precision result of the solution at medium concentration is presented in **Table 2**, and it was shown that the RSD values of retention time were less than 1%, while the RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision(Intra 4 h six injections, inter 6 days).

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (RSD values of the retention time and peak area were both less than 3%).

The LOD and LOQ were found to be 0.6295 and 1.907 µg/ml, respectively for PTN and 0.342 µg/mL and 1.038 µg/mL, respectively for HTN.

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of the markers in ternary mixture and formulations. No interferences were observed as shown in overlaid chromatograms of standard solution containing the two compounds. Good resolution and absence of interferences between the drugs determined are shown in **Figure 4.**

The robustness study indicated that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98 % and 102 % for PTN and HTN. Therefore, it can be concluded that the method is consistent for selected column and solvent brand.

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (**Table 4**). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

As shown in **Table 3**, the recovery of the investigated components ranged from 98.14 % to 100.23 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

***Applicability of the developed method in formulations***

The developed methods were applied to the simultaneous determination of PTN and HTN in the herbal formulation (Nirocil tablet) and the results are presented in **Table 5**. It was also observed that the content of PTN and HTN were comparable in the different batches of the same formulation. Therefore, the simultaneous determination of these two active components in herbal tablets could be successfully applied to improve the safety and quality control of the formulations available in the market.

The WHO has emphasized the need to develop the quality control parameters for herbal products and in this connection the present method of estimation of PTN and HTN content of the formulation is very useful. The methods are simple, precise and accurate method suitable for the routine analysis in pharmaceutical preparations.

***Comparison of Proposed methods***

The assay results for PTN and HTN in their formulation obtained by using first derivative spectrophotometry and LC methods were compared by applying the analysis of variance (ANOVA) between groups test. The calculated F-values were 0.34 for PTN and 0.04 for HTN, which were less than the tabulated F-values (5.31 for PTN and HTN). Therefore, no significant difference was found in the contents of PTN and HTN determined by the proposed LC and First derivative spectrophotometry methods.

**CONCLUSION**

Two methods were developed for the determination of HTN and PTN in the presence of each other based on different analytical techniques. Both methods were validated and found to be simple, sensitive, accurate and precise and can be applied for the simultaneous determination of PTN and HTN. Since proprietary herbal medicines containing Phyllanthus is increasingly becoming popular as a hepatoprotective in the global market, method for standardization of those medicines are in demand. The contents of the lignans can significantly vary due to a number of reasons in the proprietary medicines. So, it is highly recommended that the determination of these lignans in the proprietary Ayurvedic medicines must be done as a

routine measurement, so as to provide a safe application to patients in clinics, and good manufacture practices.

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